Neuron, Vol. 14, 1257–1264, June, 1995, Copyright © 1995 by Cell Press

Tubulin Dynamics in Neuronal Axons of Living Zebrafish Embryos

Sen Takeda, Takeshi Funakoshi, and Nobutaka Hirokawa
Department of Anatomy and Cell Biology
Faculty of Medicine
University of Tokyo
Hongo, Bunkyo-ku, Tokyo 113
Japan

Summary

The mechanism of cytoskeletal protein transport, especially the question of what kind of form the cytoskeletal proteins assume during transport in neurons in situ, has been an important, as yet unsettled issue. To clear up this matter, we adopted the embryonic zebrafish as a living animal model and applied the fluorescence recovery after photobleaching (FRAP) method. The zebrafish embryo is appropriate for this kind of study because of its transparency during the early developmental stage, allowing the observation of neurons that incorporate the microinjected fluorescent tubulin directly under fluorescence microscopy. FRAP revealed no movement of the bleached zone proximodistally, where fluorescence recovered gradually (recovery half-time, 44.2 ± 11.2 min; n = 36), suggesting that the polymers are stationary but dynamic and that the true moving form could be small oligomers or heterodimers.

Introduction

Since axons and synapses do not possess the protein synthesis machinery, most of the proteins for maintaining these structures need to be transported down the axon after being manufactured in the cell body. Of these transport mechanisms, the fast flow conveys proteins as membranous organelles, whereas the slow flow engages in transporting proteins such as cytoskeletal components (Grafstein and Forman, 1980; Hirokawa, 1991). In this sense, the slow axonal transport is indispensable for axonal elongation and maintenance of the neuronal architecture.

In fact, slow axonal transport has been extensively investigated from the time of the pulse-labeling experiment (Black and Lasek, 1980) to the recent fluorescence recovery after photobleaching (FRAP; Lim et al., 1990; Okabe and Hirokawa, 1990) or the fluorescence decay after photoactivation (FDAP; Reinsch et al., 1991; Okabe and Hirokawa, 1992). Through the course of the evolution of the experiments on slow axonal transport, the fact that the cytoskeletal components are dynamic structures has been taken as a matter of course. However, the exact mechanism of slow axonal transport, including the form of the protein during transport, is still unsettled. Two opposing hypotheses (for review, see Hirokawa and Okabe, 1992; Nixon, 1992; Hirokawa, 1993) on this problem are the so-called polymer sliding model (Lasek, 1986) and one stating that small oligomers or monomers are a transported form (Nixon and Longvinenko, 1986; Okabe and Hirokawa, 1990). Although various kinds of techniques have been attempted, they have failed to bring new insights or to uncover critical determinants for solving this question with any consistency. From another point of view, and this is vital, no experiment has been performed on neurons in a living vertebrate in situ. After a virtual avalanche of in vitro studies has been published, one of the remaining tasks regarding slow axonal transport is to analyze this phenomenon in the most physiological condition, that is, in a living animal.

With this in mind, it is clear that an ideal strategy for achieving this would be to use a zebrafish embryo as a living animal model on which studies using a fluorophore as a probe could be performed. The zebrafish (Brachydanio rerio) possesses many ideal features for this purpose. It is transparent during the embryonic stage, and the developmental fate maps have been relatively well documented (Kimmel et al., 1990; Strehlow and Gilbert, 1993; Strehlow et al., 1994). In addition, the embryonic zebrafish spinal cord has less than 20 neurons per hemisegment, which have already been well described with respect to morphology, topology, and development (Myers et al., 1986; Bernhardt et al., 1990; Kuwada et al., 1990). By microinjecting fluorescently labeled tubulin into the appropriate blastomeres of zebrafish eggs during development, which are destined to be a part of the nervous system (Strehlow et al., 1994), relatively specific incorporation of labeled tubulin into neurons can be expected. Because of the transparency of the embryo, we can identify the neurons incorporating the fluorescently labeled tubulin in situ in the living embryo without any kind of intervention, such as surgical manipulation. We took advantage of these favorable features and performed the well-established FRAP experimental run to reveal the dynamics of tubulin in situ and then compared the obtained data with the previous in vitro data (Okabe and Hirokawa, 1992) to understand better the mechanism of slow axonal transport.

Results

Characterization of Fluorescently Labeled Tubulin

It is an essential prerequisite to check the state of the fluorescently labeled tubulin to determine whether the free dye has been completely eliminated, since the more diffusible small molecular weight fluorophore might hinder the accurate assessment of the FRAP experimental run or affect the neuron under investigation by generating free radicals. We performed SDS gel electrophoresis to confirm this point. As shown in Figure 1A, there is a distinct single band on a Coomassie Brilliant Blue–stained gel (lane 1) without any kind of drift of the band, suggesting that chemical modification of tubulin did not alter the structure of the protein to any appreciable extent. Under ultraviolet light, no other fluorescent bands could be identified

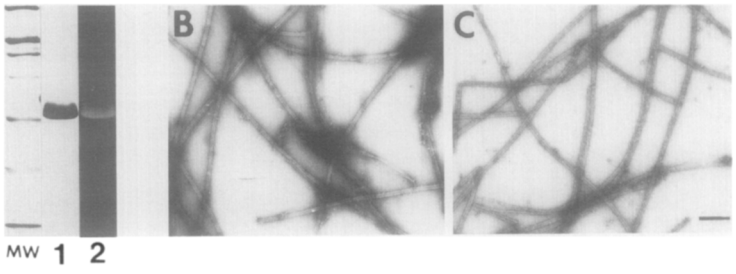


Figure 1. Characterization of Fluorescently Labeled Tubulin

(A) SDS–PAGE analysis of the fluorescently labeled tubulin reveals a distinct single band at the level of 55 kDa (lane 1). Under ultraviolet illumination, only a single band corresponding to that of Coomassie Brilliant Blue–stained gel is visualized (lane 2). Molecular weight standards (from top to bottom) are 200 kDa, 116 kDa, 92 kDa, 66 kDa, 42 kDa, 31 kDa.

(B) Negative staining of the reassembled tubulin being labeled with 5(6)-carboxy-fluorescein succinimidyl ester. Smooth-surfaced filaments can be observed, providing evidence that the capability of tubulin to polymerize itself into microtubules after tagging the fluorophore is not lost.

(C) Control assembled only from native tubulin. Bar, 200 μm.

(lane 2), confirming the fact that the free dye had been completely eliminated from the sample used in this study.

Furthermore, we carried out an in vitro reconstruction experiment to confirm that the chemically modified tubulin retained its capability to polymerize into microtubules (MTs). The fluorescently labeled tubulin was polymerized into MTs under conditions suitable for polymerization and was negatively stained with 3% uranyl acetate. The appearance of the polymerized fluorescein tubulin seemed to be normal (Figure 1B), and no stubby fragment of MTs existed. Furthermore, the appearance of the fluorescently labeled MTs mimicked that of native MTs (Figure 1C). All these facts suggested that the chemically modified tubulin did not lose its polymerizing ability.

Evaluation of Photodamage

It is necessary to predetermine the extent of energy that is absorbed by cells under mercury lamp illumination for long durations or under a argon laser beam at focused sites on the neurites, since Vigers et al. (1988) reported that microtubules labeled with fluorescein break up under such illumination. Here we performed three sets of experiments to fix this matter.

First, fluorescently labeled tubulin was polymerized with rhodamine-labeled and native tubulin as described previously (Okabe and Hirokawa, 1993). Microtubules formed by this regime were initially observed under a rhodamine band pass filter, and the images were recorded. Then, the same field was observed under a fluorescein filter for 5 s, about the same duration as that to be used for the zebrafish embryo during the course of the FRAP experimental run. After incubation for 10 min, the same field was observed under a rhodamine band pass filter, and the image was recorded. The two pictures before and after mercury lamp illumination were compared to determine whether there were any morphological changes in the filament pattern. As can be seen in Figures 2A and 2B, there were no remarkable differences between the two states. Since the in vitro system is far more susceptible to damage by mercury lamp illumination than the living animal in situ, as there is no buffer action attenuating the effects of free radicals, it is conceivable that the relatively short duration of mercury lamp illumination applied to the embryonic zebrafish neurons in situ would not influence the viability of the cells.

Second, we evaluated the effect of mercury lamp illumination and argon laser beam application on the mouse dorsal root ganglion (DRG) neurite. The DRG plated onto a laminin-coated coverslip was microinjected with fluorescently labeled tubulin, and after incubating the cell for up to 22 hr, the small part of the neurite incorporating the fluorescently labeled tubulin was marked with the argon laser beam. After incubation for 10 min, the cell was fixed with Karnovsky's fixative for 30 min, followed by a series of procedures for electron microscopy. Identification of the cell being photobleached was made by dint of the coverslip, on which a kind of grid pattern was etched (Cellocate, Eppendorf). There was no deformity of the cell after bleaching the particular small portion of the neurite (Figures 3B and 3D). By electron microscopy, the neurite being illuminated with laser for 1 s appeared to be normal, with special reference to the fine structure, especially in the continuity of the microtubules between the illuminated and nonilluminated region (Figures 3E, 3F, 3G, and 3H). The microtubules extending along the neurite had a smooth surface. No accumulation of vesicles or other peculiar structures as evidence of photodamage were identified. All in all, the energy level applied to the DRG, the same level as also used for the experimental run in the living

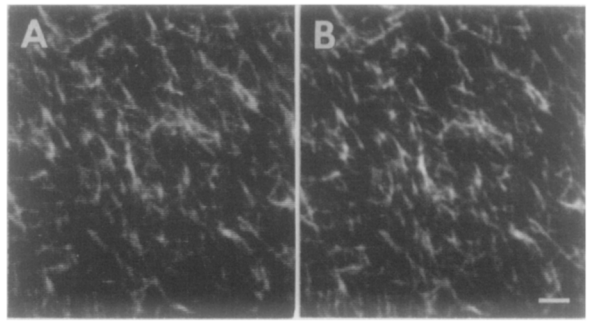


Figure 2. Evaluation of Photodamage in In Vitro Reconstruction System

Fluorescently and rhodamine labeled tubulin was reassembled with native tubulin and observed under a 100 W mercury lamp for 5 s with the use of a band pass filter for fluorescein. The filament pattern between preillumination (A) and postillumination (B) recorded under the rhodamine band pass filter is almost identical. Bar, 10 μm.

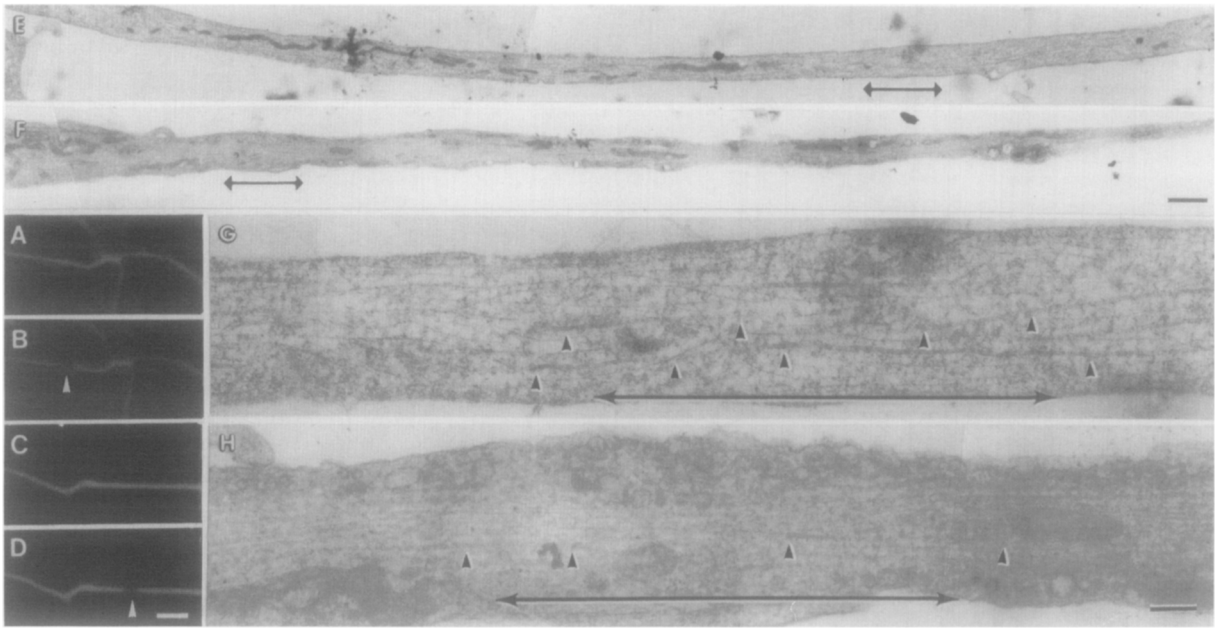


Figure 3. Electron Microscopical Evaluation of the Influence of a 100 W Mercury Lamp and Laser Illumination on Mouse DRG Cells

The gross appearance of neurites 10 min after photobleaching does not show any alteration (B and D). (A) and (C) depict the state before the run. (E) and (F) correspond to (B) and (D), respectively. No abnormal images are identified by electron microscopy (E–H). (G) and (H) are magnified images of (E) and (F), respectively. Note that the microtubules in the axon of the bleached area are continuous with those of the unbleached area (arrowheads). In addition, the whole neurite being illuminated by the mercury lamp shows no pathological alteration, suggesting that the level of energy adopted in our experiment was appropriate for investigating the dynamic demeanor of tubulin. Closed arrows in (E–H) point to the sites at which photobleaching was carried out.

Bars, 5 μm (A–D); 1 μm (E and F), 200 nm (G and H).

zebrafish embryo, might not have had a deleterious effect on the viability of the cell.

Finally, the whole embryo on which a course of FRAP (~2 hr) was run was fixed for electron microscopic observation. Then, serial sections of the whole embryo were cut with glass knives longitudinally, and they were stained with toluidine blue. The preparations were observed under a conventional light microscopy to identify the neurites of our objective. With identification of the neurites on which the FRAP had been performed, the sections were reembedded in Epon. Ultrathin sections were cut and observed by electron microscopy. Figure 4 shows examples of the image of the embryonic zebrafish neurites just before (Figure 4A) and at 10 min after photobleaching (Figure 4B), in which the morphology of the neurite does not show any kind of alteration. Electron microscopical observation of the photobleached area (Figures 4D, 4E, 4F, and 4G) showed no perturbation of organella, including the microtubules. In addition, there were no abnormal structures or vesicle accumulation in the vicinity of the bleached area.

From all the above results, it was concluded that the energy level adopted in this study did not exceed the limit above which photodamage might occur.

FRAP on the Embryonic Neuron of the Zebrafish

The zebrafish embryos microinjected with fluorescently labeled tubulin after incubation of 20–24 hr were observed under an orthostatic microscope equipped with a 100 W mercury lamp. Of all the embryos injected (~2000), 3%–4% incorporated the fluorescent tubulin specifically into neurons. We chose the embryos of brightly labeled, well-isolated neurons. We made identification of the neurons according to previous studies (Myers et al., 1986; Bernhardt et al., 1990; Kuwada et al., 1990). The embryos were mounted on glass slides within a droplet of 2% methyl cellulose to hinder spontaneous movement, and they were sealed with coverslips. Under this condition, the embryos were still alive for more than 1 day while displaying normal development. Observation of the mounted embryos was carried out by using a water immersion objective. This lens has a wide working distance (~7 mm), enabling the observation of the embryos without applanation.

Prior to FRAP experimental run proper, we monitored the growing rate of the axons meticulously. As can be seen in Figure 5, the growing tip of the Rohon-Beard (RB) neuron axon moved in the caudal direction (arrowheads), whereas the reference point (arrow, nucleus of a nonneuronal cell) still remained at the same place. Although the growing tip was out of a visual field at 30 min, the axon under observation grew at least 10 μm. Therefore, the growing velocity of it was calculated to be more than 20 μm/hr. This value verifies our FRAP data, showing that the neurons under investigation extend their axons without any token of perturbation.

After choosing neurons for the study, a small portion of the neurites was illuminated with the argon laser beam for 1 s, within the limit that would not cause photodamage as established in our study. Photobleaching marks were

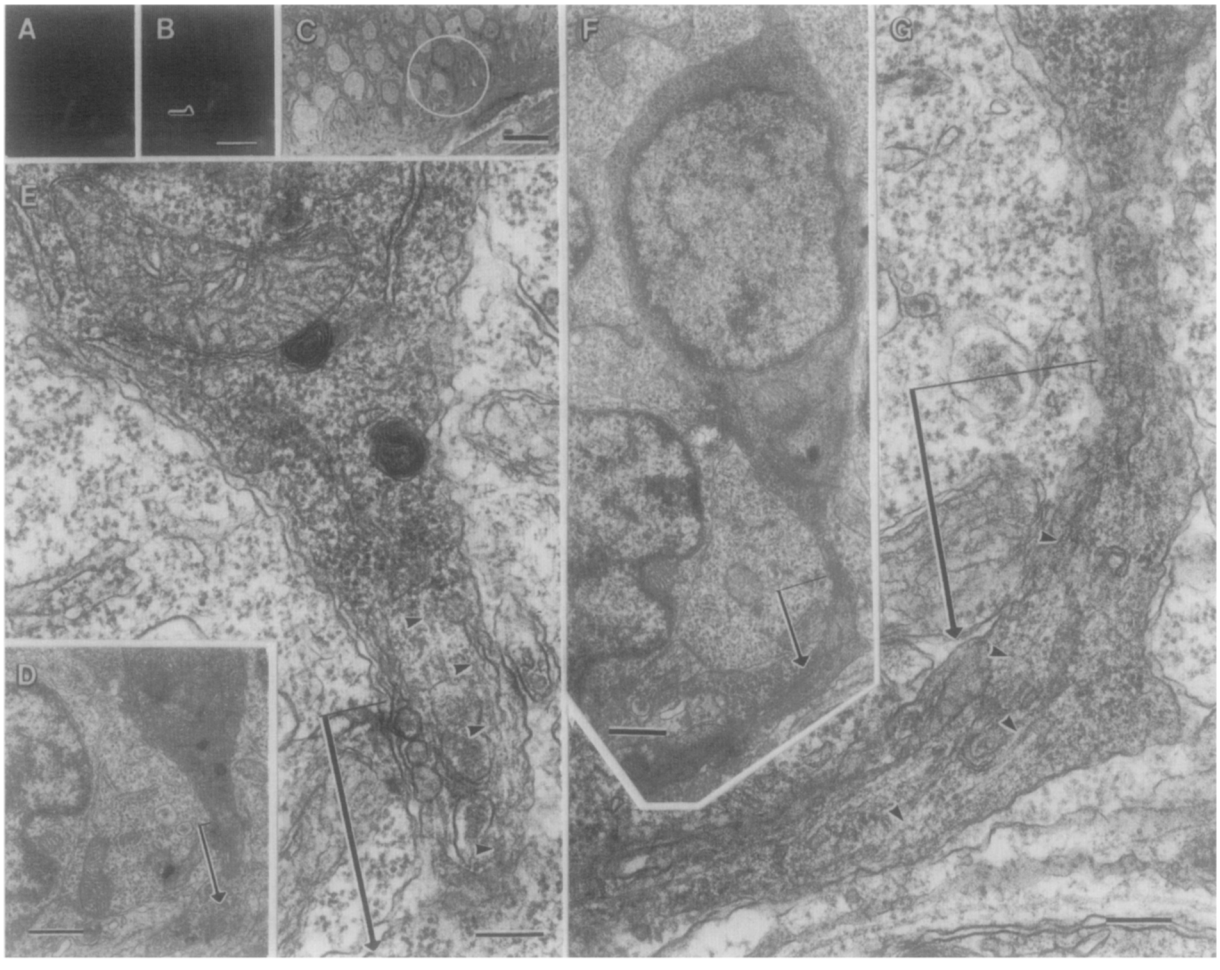
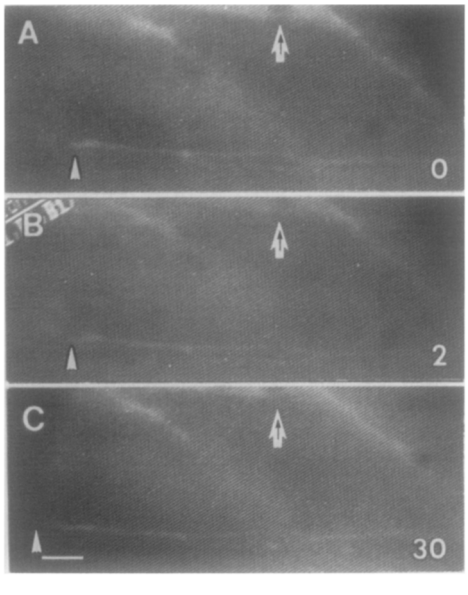


Figure 4. In Situ Analysis of Photodamage of Neurons in Embryonic Zebrafish Spinal Cord

Lateral view fluorescent image of tubulin incorporating interneuron being processed for electron microscopical investigation before (A) and 10 min after (B) photobleaching. (C) shows light microscopical serial section (toluidine blue stained) for identification of the neuron being photobleached, which is then processed for analysis by electron microscopy. The encircled neuron corresponds to that shown in (B). (D) and (F) are serial electron microscopical sections of the cell shown in (C). No alterations in the structure of the cytoskeletal components can be identified in (E) and (G), which show the bleached region (arrows) in detail. Arrowheads indicate continuous intact microtubules. All panels are in sagittal plane, with rostral at right. Bars, 10 μm (A–C); 1 μm (D and F); 400 nm (E and G).



made on the neurites of both RB cells (n = 11) and motoneurons in the spinal cord (n = 25). For the former, we performed FRAP on the neurites at a distance of 10–40 μm from the somata, and for the latter, at more than 30 μm. The images after photobleaching were recorded intermittently at ~ 30 min intervals. Figures 6A–6D and Figures 7A–7D depict two examples of the FRAP experimental run in the case of RB neurons. During the course of the experiment, there was no deformity with respect to the gross appearance of the neurites being photobleached. At 35 or 39 min after photobleaching, the marks recovered their fluorescent intensity to a certain extent (Figure 6C;

Figure 5. Monitoring of Growing Axon In Situ

The axonal tip (arrowheads) grows more than 10 μm in 30 min with reference to the stationary nucleus of nonneuronal cell (arrows). Elapsed time in minutes is labeled at the bottom right corner of the figure. Bar, 10 μm.

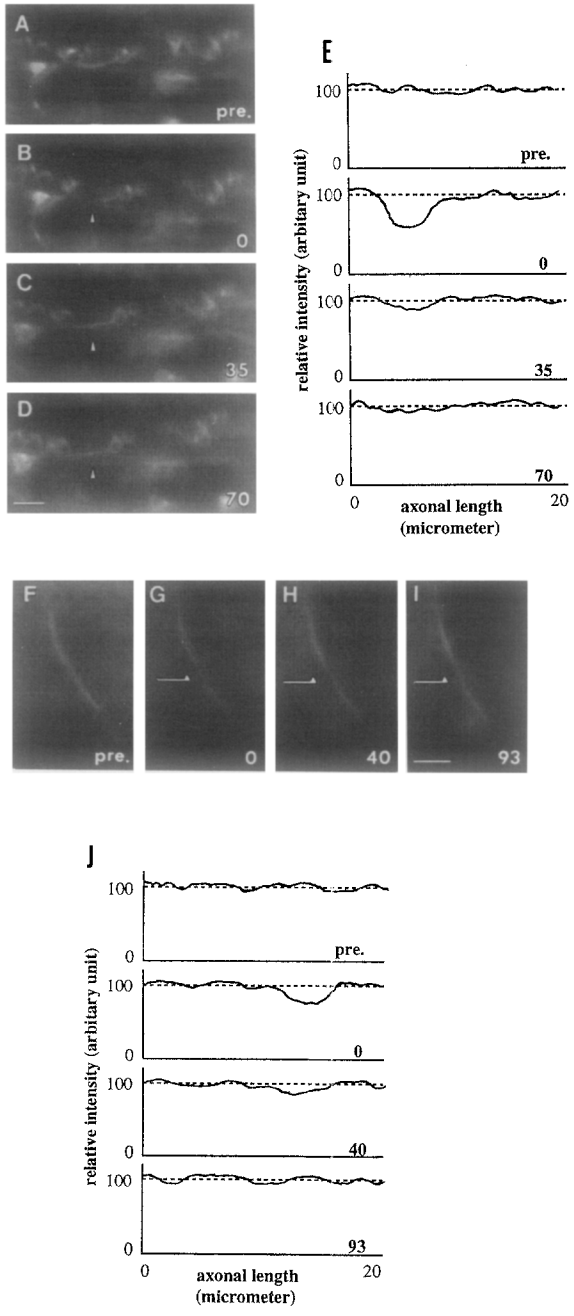


Figure 6. FRAP Run on Spinal Cord Neurons in the Living Zebrafish Embryo In Situ

The whole embryo is mounted on a glass slide and observed under a microscope equipped with a 100 W mercury lamp. Fluorescent images of both a RB neuron (A–D) and axon of the spinal cord motoneuron (F–I) were recorded intermittently. The bleached area is stationary in both series. Arrowheads indicate the bleached zone, and the elapsed time in minutes is indicated in the bottom right corner of the panels. Quantitative measurements of the bleached zone are presented alongside each fluorescent panel (E and J). Bar, 10 μm.

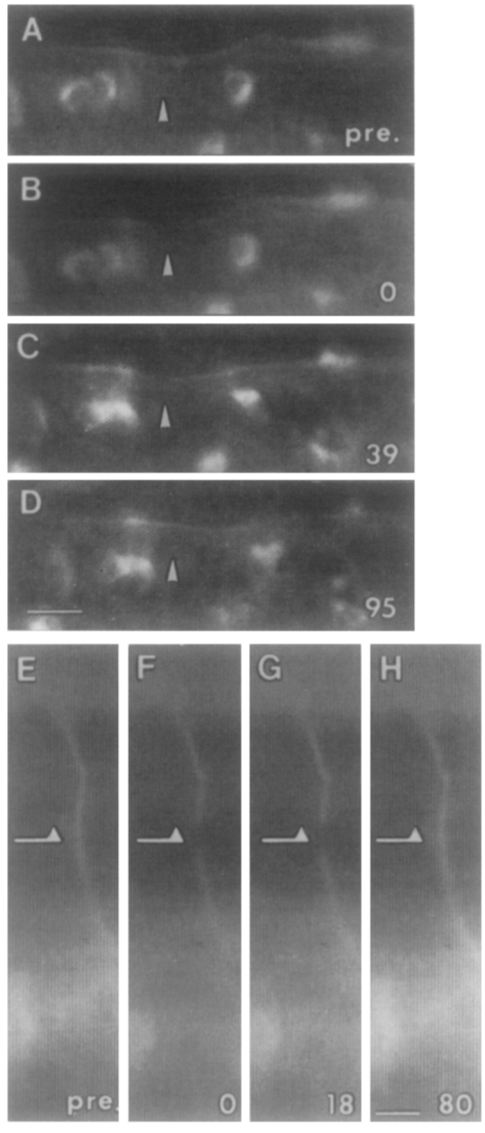


Figure 7. Another Example of FRAP on the RB Cell and Motoneuron

The bleached area is stationary in both series (RB cell [A–D]; motoneuron [E–H]). Arrowheads indicate the bleached zone, and the elapsed time in minutes is labeled at the bottom right corner of the panels. All panels are in sagittal plane, with rostral at right. Bar, 10 μm.

Figure 7C). The intensity profile corresponding to the panel indicates that the bleached area restored its fluorescence by more than 50% compared with the state soon after photobleaching (Figure 6E). At 70 (Figure 6D) or 95 min (Figure 7D), the bleached regions could no longer be identified because of complete fluorescence recovery. In the experiments using this type of neuron, the marks did not translocate in any of the trials (n = 11), suggesting that the form of tubulin traveling down the axon might be a small oligomer (heterodimer) or a monomer, but not a polymer. The intensity profiles (Figure 6E) also provide evidence that no translocation of the bleached regions occurred. We performed the same FRAP experiment on the relatively long extending neurites of the spinal cord motoneurons (n = 25). The results were quite similar to those of RB neurons. The bleached marks did not translocate in any direction, and the fluorescence recovered gradually (Figures 6F–6I; Figures 7E–7H). The intensity profile for Figures 6F–6I, in which the measurements were carried out along the neurite, also gives credit for that (Figure 6J)

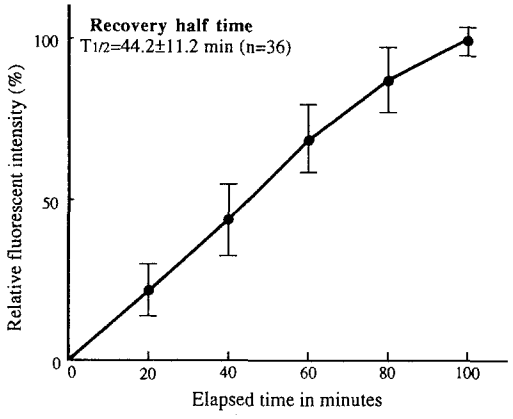


Figure 8. Graphic Presentation of the FRAP Experimental Run

Relative fluorescence intensity of the bleached area was plotted against time. The bars indicate standard deviation. Averaged recovery half-time calculated from 36 experimental runs is shown at the top of the figure.

As a summary of our FRAP experiment, we analyzed the data from both RB cells and motoneurons to draw a fluorescence recovery graph (Figure 8), in which the parameter termed recovery half-time ($T_{1/2}$) (Okabe et al., 1993; Takeda et al., 1994) was determined to be 44.2 ± 11.2 min (mean ± SD; n = 36). Because there were seemingly no differences in the time course of fluorescent recovery between the two cell types, we calculated this value from the combination of the data from these two cell types. This value is of importance, in that it indicates how fast the local turnover of tubulin between the soluble pool and the assembled MTs takes place. In addition, Figure 8 can reinforce our oligomer model for tubulin transport by providing the following explanation; if the tubulin has been transported down the axon as polymers, the recovery of fluorescent intensity might have shown a more abrupt rise during the initial phase, because bulk replacement of the tubulin would have occurred at the bleached region. Therefore, the gradual recovery seen in Figure 8 also supports our oligomer model.

Discussion

Zebrafish as a Tool for Studying Slow Axonal Transport

In the field of genetics, zebrafish are widely used for searching for mutations that disturb development (for review, see Mullins et al., 1994). However, in the field of cell biology, there are few reports in which they are used as an experimental animal. Because procedures such as FRAP or FDAP utilize fluorescence, transparency of the animal is one of the most important factors for performing experiments in the living individual in situ.

Transparency during their embryonic stage, a relatively short life cycle, and ease in keeping and handling (Westerfield, 1989) all attracted us to the use of zebrafish for this study. Furthermore, the total number of neurons existing at ~24 hr postfertilization is reported to be at most 20 (Bernhardt et al., 1990; Kuwada et al., 1990), enabling us to identify the neurons easily. Another favorable feature of this animal is that the pressure-directed inundation of tubulin into the axon can be avoided because microinjection is performed at the stage of early development, as in the case of Xenopus eggs (Tanaka and Kirschner, 1991; Reinsch et al., 1991; Okabe and Hirokawa, 1992). Therefore, we microinjected the fluorescently labeled tubulin into blastomeres at the 16–32 cell stage. At this stage, the fate of each blastomere is relatively determined, and the incorporation of fluorescently labeled tubulin into the axon might be achieved more specifically than by injecting at the time of the 1–2 cell stage (Strehlow et al., 1994). To avoid any capriciousness of incorporation (that is, uptake of fluorescent tubulin into tissue other than neurons), it would actually be advisable to perform microinjection at a more advanced stage, such as 128 cells or more, but technical limitations prevented us from doing so.

The Mechanism of Slow Axonal Transport

One of the most important questions in the field of slow axonal transport is at least partly answered in this study. Although it would be premature to draw definitive conclusions from our results in zebrafish embryonic neurons, our data suggest a solution to the question of how cytoskeletal proteins are transported and behave in the living vertebrate. The form of tubulin traveling down the axon is a heterodimer or small oligomer, and tubulin molecules once incorporated into the preexisting microtubules do turn over within a relatively short half-time (Figure 8), as reported previously in the cultured neurons (Lim et al., 1989, 1990; Reinsch et al., 1991; Okabe and Hirokawa, 1990). However, our study did not reveal how much of the tubulin is interchangeable. Furthermore, the animal employed in our study was in a developmentally earlier stage, in which the modification of tubulin, which modifies the state of acetylation or tyrosination (Ahmad et al., 1993), displays a slightly different grade compared with mature quiescent neurons, leaving the possibility that the turnover of tubulin might be estimated as being faster than mature, full-fledged neurons (Lim et al., 1989; Nixon and Shea, 1992). In a previous study, we roughly calculated the recovery half-time of tubulin in adult mouse DRG to be 45–90 min (Okabe and Hirokawa, 1990). This period is slightly longer than that of our present study (~44 min), probably resulting more from the difference in age of the animal when the experiment was carried out than a difference in species. Since we used an energy level at which no photodamage could be caused, we believe that the results can be accepted as a genuine phenomenon taking place in living neurons.

For the present, we should like to devote our consideration to the following two matters: first, the axonal growing rate during the experiment; and second, slow axonal transport among different species.

First, the estimation of growing rate of the neurites under investigation is of great importance, because we cannot rule out the possibility that very slow or no growing of the neurites might have led to the lack of tubulin movements in this system. The measured growing rate of the RB cell axon was more than 20 μm/hr (see Figure 5), which is

compatible with the estimated value (~30 μm/hr) according to Kuwada et al. (1985). Regarding the spinal motoneurons, monitoring of the growth cone extension is hardly possible because of its anatomical features. Nevertheless, it was possible to make an estimation of axonal elongation 19–27 hr after fertilization according to the procedure of Myers et al. (1986), which gave a result of 10 μm/hr. Combining our data and the estimation of others, we can state that the neuronal cells in the present study definitely extended their axons at a rate of 10–50 μm/hr in toto. This further substantiates our FRAP data.

The second question is, can we extrapolate the data obtained in the fish to other vertebrates or other animals having well-differentiated neurons. In our previous study (Okabe and Hirokawa, 1992), the behavior of microtubules in the axon was seemingly contradictory, being stationary in mouse DRG and sometimes translocated proximodistally in Xenopus motoneurons. In that case, we explained that the pulling of whole axoplasms by growth cones in the cultured Xenopus neurons is responsible for this seemingly opposing phenomenon (Okabe and Hirokawa, 1992). From another standpoint, the cultured Xenopus neuron is considered to be a special case and does not seem to reflect the real phenomenon taking place in vivo. Considering that the growth rate of the zebrafish axon is nearly the same as that for the mouse DRG cells (Okabe and Hirokawa, 1993), as mentioned above, our present results seem quite reasonable. From these two considerations, we conclude that the results of our present study in zebrafish are of significance in showing that the form of tubulin traveling down the axon is possibly a heterodimer or a small oligomer, but not a polymer, among vertebrate neurons.

Finally, because mouse DRG extends processes longer than 1 μm, mere diffusion cannot explain the force for transport of tubulins (Okabe and Hirokawa, 1990, 1993). Future study to reveal an actively moving form of tubulin directly is necessary and is under way in our laboratory.

Experimental Procedures

Animals

We collected spawned eggs of zebrafish (Brachydanio rerio) just after fertilization. The zebrafish were a generous gift from Dr. H. Okamoto, Department of Physiology, Faculty of Medicine, Keio University. We also used commercially available ones and kept them according to Westerfield (1989). Throughout the course of the experiments, we referred to the staging diagram proposed by Westerfield (1989).

For evaluating the effect of laser irradiation on neurites of the zebrafish embryos, we used the cultured DRG cells of mice C57 BL. The details of the culture process were previously described (Okabe and Hirokawa, 1992; Takeda et al., 1994).

Preparation and Characterization of Labeled Tubulin

Purified porcine tubulin was labeled with 5(6)-carboxyfluorescein succinimide (Molecular Probes, Eugene, OR) as previously described (Kellog et al., 1988). The purity of the labeled fluorescent tubulin was checked by SDS-PAGE. The polyacrylamide gel was illuminated under an ultraviolet illumination to confirm that the unbound dye was completely eliminated from the preparative sample. Furthermore, for verification of the assembly competence after labeling, the labeled protein was assembled into microtubules in vitro (Okabe and Hirokawa, 1992), stained negatively with 3% uranyl acetate, and then viewed under electron microscopy as previously described (Takeda et al., 1994).

Optical Installment

Fundamentally, the optical system used in this study was almost the same as used in the previous studies (Okabe and Hirokawa, 1992; Takeda et al., 1994). However, for the convenience of observing the whole embryo under the fluorescence mode, we used an Olympus orthostatic microscope BH-2. As an objective lens for observation of the zebrafish embryos, we used a water immersion lens with a longer working distance (~7 mm, WPlan FL 40 UV, Olympus, Hachioji, Japan). Other optical alignments, including the light pathway for the argon laser, were the same as those in our previous studies.

Evaluation of Photobleaching on the Polymerized Microtubules Labeled with Fluorescein

Evaluation of photodamage in the in vitro reconstruction system was done by the following scheme. Fluorescently and rhodamine labeled tubulins were reassembled with native tubulin and illuminated under a 100 W mercury lamp for 5 s with the use of a band pass filter for fluorescein. Observation of the tubulin of both pre- and postillumination states was done with a rhodamine band pass filter.

Microinjection of Fluorescent Tubulin

Microinjection was carried out both on cultured mouse DRG cells and developing zebrafish eggs. As for the DRG, the procedure followed was similar to that of our previous study (Takeda et al., 1994).

Microinjection into the developing zebrafish eggs was carried out on blastomeres of the 16–32 cell stage, especially on those considered to contribute to part of the central nervous system. A glass capillary with a larger tip diameter (~5 μm) than that for microinjection of cultured cells was used. The egg (at 16–32 cell stage) with chorion was mounted on a hollow created in 1.2% agar dissolved in embryo medium (Westerfield, 1989) and was microinjected with fluorescently labeled tubulin. The injected eggs were then collected, placed in the embryo medium, and incubated for about 24 hr. We injected fluorescently labeled tubulin into ~2000 embryos. About 70% of the injected animals survived and about 5% of the survivors (~70) had fluorescently labeled neurons. Of these neurons, we chose well demarcated ones (n = 36) for the FRAP runs.

FRAP on Zebrafish Embryos and Mouse DRG

The embryos microinjected with fluorescently labeled tubulin that reached the stage of 20–25 hr postfertilization were dechorionated and mounted on a glass slide, embedded in 2% methyl cellulose, and finally sealed with a coverslip without destroying the embryos. The mounted embryo was observed under fluorescence microscopy to detect the neurons incorporating the fluorescently labeled tubulin. After confirming that the well-formed neurons were brightly labeled, a 488 nm argon laser was applied to a small portion of the neurite. During the course of fluorescence recovery after photobleaching, the images of the cells were recorded on ARGUS-100 (Hamamatsu Photonics, Hamamatsu, Japan) intermittently and stored in a Sony U-matic video cassette tape recorder (model VO-9600; Sony, Tokyo, Japan). Upon completion of the observation, the embryos were fixed chemically for electron microscopy as described in the next section. Concerning the same experimental run for DRG, we followed the procedure of the previous study (Takeda et al., 1994).

Electron Microscopy

The zebrafish embryos used for FRAP or observed under a mercury lamp (100 W) for detection of fluorescence were fixed immediately after the experiments. They were immersed in fixative containing 3% glutaraldehyde, 2% paraformaldehyde, 1% acrolein, and 1% dimethyl sulfoxide in PBS (pH 7.4) for ~2 hr (Kuwada et al., 1990, with slight modification) and washed with PBS for 3 min. Then, the yolk sac of the embryo was eliminated with a pair of electrolytically sharpened tungsten needles, followed by fixation with 1% osmium tetroxide in cacodylate buffer (pH 7.4) for 2 hr. Block staining by 1% methanolic uranyl acetate was carried out overnight, and the specimens were dehydrated through a series of graded concentrations of ethanol. After this, the whole embryo was embedded in Epon (Polyscience, New York). The Epon bloc was trimmed according to information from the bleached neurite by counting the number of somites and by referring to images of the neurons. The Epon bloc was initially serially sectioned with a glass knife, and after confirming the neuron upon which the

photobleaching procedure was carried out, we reembedded it into Epon and processed it for ultrathin sections (Schabtach et al., 1974).

Acknowledgments

Correspondence should be addressed to N. H. We thank Dr. S. Okabe for helping us in establishing this experimental system; Dr. H. Okamoto for supplying us with the wild-type zebrafish and giving us various useful suggestions; Mr. M. Naito and Mr. T. Nagano at Olympus Optics (Hachioji, Tokyo) for their cooperation in microscope development used in this experiment; Dr. Y. Okada for valuable discussions and technical suggestions; Mrs. Y. Kawasaki and Mrs. H. Sato for their secretarial assistance. This work was supported by a Special Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan, and a grant from Riken to N. Hirokawa.

The costs of publication of this aricle were defrayed in part by the payment of page charges. This article must therefore be hereby marked *"advertisement"* in accordance with 18 USC Section 1734 solely to indicate this fact.

Received November 21, 1994; revised March 27, 1995.

References

Ahmad, F. J., Pienkowski, T. P., and Baas, P. W. (1993). Regional differences in microtubule dynamics in the axon. J. Neurosci. *13*, 856–866.

Black, M. M., and Lasek, R. J. (1980). Slow components of axonal transport: two cytoskeletal networks. J. Cell Biol. *86*, 616–623.

Bernhardt, R. R., Chitnis, A. B., Lindamer, L., and Kuwada, J. (1990). Identification of spinal neurons in the embryonic and larval zebrafish. J. Comp. Neurol. *302*, 603–616.

Grafstein, B., and Forman, D. S. (1980). Intracellular transport in neurons. Physiol. Rev. *60*, 1167–1283.

Hirokawa, N. (1991). Molecular architecture and dynamics of the neuronal cytoskeleton. In Neuronal Cytoskeleton, R. D. Burgoyne, ed. (New York: Wiley Liss), pp. 5–74.

Hirokawa, N. (1993). Axonal transport and the cytoskeleton. Curr. Opin. Neurobiol. *3*, 724–731.

Hirokawa, N., and Okabe, S. (1992). Microtubules on the move? Curr. Biol. *2*, 193–195.

Kellog, D. R., Mitchison, T. J., and Alberts, B. M. (1988). Behaviour of microtubules and actin filaments in living Drosophila embryos. Development *103*, 675–686.

Kimmel, C. B., Warga, R. M., and Schilling, T. F. (1990). Origin and organization of the zebrafish fate map. Development *108*, 581–594.

Kuwada, J. Y., Bernhardt, R. R., and Nguyen, N. (1990). Development of spinal neurons and tracts in the zebrafish embryo. J. Comp. Neurol. *302*, 617–628.

Lasek, R. J. (1986). Polymer sliding in the axons. J. Cell Sci. (Suppl.) *5*, 161–179.

Lim, S. S., Sammak, P. J., and Borisy, G. G. (1989). Progressive and spatially differentiated stability of microtubules in developing neuronal cells. J. Cell Biol. *109*, 253–263.

Lim, S. S., Edson, K. J., Letourneau, P. C., and Borisy, G. G. (1990). A test of microtubule translocation during neurite elongation. J. Cell Biol. *111*, 123–130.

Mullins, M. C., Hammerschmidt, M., Haffter, P., and Nüsslein-Volhard, C. (1994). Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. Curr. Biol. *4*, 189–202.

Myers, P. Z., Eisen, J., and Westerfield, M. (1986). Development and axonal outgrowth of identified motoneurons in the zebrafish. J. Neurosci. *6*, 2278–2289.

Nixon, R. A., and Longvinenko, K. B. (1986). Multiple fates of newly synthesized neurofilament proteins: evidence for a stationary neurofilament network distributed nonuniformly along axons of retinal ganglion cell neurons. J. Cell Biol. *102*, 647–659.

Nixon, R. A. (1992). Slow axonal transport. Curr. Opin. Cell Biol. *4*, 8–14.

Nixon, R. A., and Shea, T. B. (1992). The dynamics of neuronal intermediate filaments: a developmental perspective. Cell. Motil. Cytoskel. *22*, 81–91.

Okabe, S., and Hirokawa, N. (1990). Turnover of fluorescently labelled tubulin and actin in the axon. Nature *343*, 479–482.

Okabe, S., and Hirokawa, N. (1992). Differential behaviour of photoactivated microtubules in growing axons of mouse and frog neurons. J. Cell Biol. *117*, 105–120.

Okabe, S., and Hirokawa, N. (1993). Do photobleached fluorescent microtubules move? Reevaluation of fluorescence laser photobleaching both in vitro and in growing Xenopus axon. J. Cell Biol. *120*, 1177–1186.

Okabe, S., Miyasaka, H., and Hirokawa, N. (1993). Dynamics of neuronal intermediate filaments. J. Cell Biol. *121*, 375–386.

Reinsch, S. S., Mitchison, T. J., and Kirschner, M. (1991). Microtubule polymer assembly and transport during axonal elongation. J. Cell Biol. *115*, 365–379.

Schabtach, E., Terry, A., and Parkening, A. (1974). A method for sequential high resolution light and electron microscopy of selected areas of the same material. J. Cell Biol. *61*, 261–264.

Strehlow, D., and Gilbert, W. (1993). A fate map for the first cleavages of the zebrafish. Nature *361*, 451–453.

Strehlow, D., Heinrich, G., and Gilbert, W. (1994). The fates of the blastomeres of the 16-cell zebrafish embryo. Development *120*, 1791–1798.

Takeda, S., Okabe, S., Funakoshi, T., and Hirokawa, N. (1994). Differential dynamics of neurofilament H protein and neurofilament L protein in neuron. J. Cell Biol. *127*, 173–185.

Tanaka, E., and Kirschner, M. (1991). Microtubule behavior in the growth cones of living neurons during axon elongation. J. Cell Biol. *115*, 345–363.

Vigers, G. P. A., Coue, M., and McIntosh, J. R. (1988). Fluorescent microtubules break up under illumination. J. Cell Biol. *107*, 1011–1024.

Westerfield, M. (1989). The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Brachydanio rerio*). (Eugene, Oregon: University of Oregon Press).